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Small-molecule inhibitors reveal an indispensable scaffolding role of RIPK2 in NOD2 signaling

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29th March 2018

Thank you for submitting your manuscript on the characterization of small RIPK2 inhibitors that modulate RIPK2 function in a RIPK2 kinase activity-independent manner. The manuscript has now been reviewed by three expert referees whose comments are provided below.

As you can see, referees #1 and 3 find the findings novel and of high interest to the field and provide constructive feedback on how to further revise your manuscript prior to publication. Referee #2 is less supportive and only offers brief comments on the study. I have looked at the comments carefully and I agree with referees #1 and 3 that the analysis adds important new insight. The referees bring up some issues that should be resolved in a revised version. In particular, they point out that the clarity of the manuscript will greatly increase if you would edit and streamline the text incorporating their suggestions. Particular attention should be given to provide key information for non-expert readers to understand your experiments and the significance thereof. Given the overall interest of your study, I would like to invite you to revise the manuscript in response to the referees' reports.

REFEREE REPORTS

Referee #1:

In this paper, Hrdinka et al. explore the mechanisms underlying the mode of action of RIPK2 inhibitors. The authors show that, surprisingly, RIPK2 kinase activity is dispensable for NOD2

signaling. Instead they convincingly demonstrate that the inhibitory mechanism relies on the disruption of XIAP binding to RIPK2 and the subsequent ubiquitination of the latter. Targeting RIPK2 might be of interest in a range of inflammatory conditions, such as multiple sclerosis and Crohn's disease. Therefore, the presented findings have important therapeutic implications and further might help to develop novel inhibitors to modulate RIPK2 signaling.
This is a well performed study that provides several interesting observations that are of interest for the wider audience of the journal. The results are straightforward and support the conclusions that are made. I have only some minor concerns that should be addressed:
- Are the novel RIPK2 inhibitors described here specific for RIPK2, as opposed to RIPK1/RIPK3?
- Can the authors show that the inhibitors are not cytotoxic.
- Why do the authors only test RIPK2-XIAP binding. What about cIAP1/2? Nachbur et al. (2015) have previously shown that WEHI-345 interferes with RIPK2-cIAP1 interaction. In fact, this should be acknowledged in the paper.
- Figure 5C: what is the extra lower band in the anti-GST blot that is missing in similar blots in Figures 5B and 5E.
- Figure 5E: Is it pull-down of recombinant proteins in vitro or with the use of cell lysate? Is the "Lysate" labeling correct?
- The manuscript sometimes lacks details necessary for the general audience (not experts in the field) to understand the experiments performed. Figures should be labeled better, for example it is not always clear which cells are used without checking figure legends.
- Proof reading for some typos is necessary, i.e. p3 first paragraph " granulatomous pathologies"; p5 second paragraph "ponatinb"

Additional suggestions that may improve the study:
- Testing new inhibitors in a relevant mouse model of inflammatory disease, where RIPK2 is implicated, would further validate the therapeutic value of the findings.

Referee #2:

Manuscript EMBOJ-2018-99372

Small-molecule inhibitors reveal an indispensable scaffolding role of RIPK2 in NOD2 signaling

By Hrdinka et al.

The authors investigated the effects of small-molecule inhibitors of RIPK2. They report that RIPK2 kinase activity is responsible for NOD2 inflammatory signaling. They went on to show that RIPK2 inhibitors function by neutralizing XIAP binding and XIAP-mediated ubiquitination of RIPK2.

Major points:
The manuscript is not within the primary focus of EMBO Journal and is therefore considered to be suitable for a more specialized journal on chemical compounds.

Referee #3:

The manuscript by Hrdinka and coworkers is of considerable interest as it provides a very thorough analysis of the mechanism by which small molecule compounds that bind to the ATP-binding pocket of RIPK2 can be used to manipulate RIPK2 function even though the kinase activity is not essential. This discovery is of considerable importance because RIPK2 is a key player in inflammatory signalling and these compounds have the potential to improve several pathologies. This is because the compounds disrupt interaction of RIPK2 with the E3 ligase XIAP, an essential step in asseble of a stable inflammatory signalling complex. It is likely that the compounds that target RIPK2 will be more selective and effective than compounds that target XIAP.

I have no major concerns, the manuscript is comprehensive and describes an elegant set of

experiments, is well-written and a pleasure to read.

Minor
1) It would be good to describe the classes of kinase inhibitiors at the outset. Different classes are referred to in the introduction (and throughout) but for those not in the field it would be helpful to know about the significance of the classes earlier.
2) Please indicate r2 in figure 3d.
3) The resolution of the structure is only 3.2A. Because the details of the active site are important it would be helpful to show the electron density for this region and comment on the quality of the map in the main text.
4) Although the compounds will be described in detail elsewhere it would be helpful to include a simple schematic alongside the crystal structure in Figure 3.

1st Revision - authors' response 7th May 2018

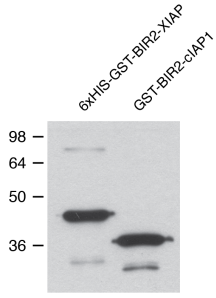
Point-to-point response to referee comments.

Referee #1:

- Are the novel RIPK2 inhibitors described here specific for RIPK2, as opposed to RIPK1/RIPK3?
The reviewer raises an important point here. We have now performed kinase assays with recombinant RIPK1 and RIPK3 and observed no inhibition of either kinase by CSLP37 and CSLP43 at concentrations up to 1 μM. The new data is shown in Figure EV2C.

- Can the authors show that the inhibitors are not cytotoxic.
We have included cytotoxicity assays with the CSLP37 or CSLP43 inhibitors and have not observed any toxicity of either in the cell types used in the study (HEKBlue, RAW264.7, U2OS, THP1 cells) treated with the compounds at concentrations up to 1 μM. The data is shown in Figure EV2B.

- Why do the authors only test RIPK2-XIAP binding. What about cIAP1/2? Nachbur et al. (2015) have previously shown that WEHI-345 interferes with RIPK2-cIAP1 interaction. In fact, this should be acknowledged in the paper.
We focussed on characterising the RIPK2-XIAP binding because XIAP is indispensable for NOD2 signalling whereas cIAP1/2 both are not needed (Stafford et al. 2018, Damgaard et al. 2013). That said, we fully agree that the previous work on WEHI-345 and its effect on IAP-RIPK2 interaction should be included in the paper. Also, since cIAPs do ubiquitinate RIPK2 (at least in the absence of XIAP), we acknowledge that it is relevant to know if the inhibitors affect the interaction of RIPK2 with cIAPs. We have therefore performed interaction studies also with a GST-tagged BIR2 domain of cIAP1. The data is shown in Figure EV4B. The pulldown experiments show (as expected) that cIAP1-BIR2 can pulldown RIPK2 from cell lysates and that CSLP37 and CSLP43 both antagonise the interaction. These data suggest that XAIP and cAIP1 interact with RIPK2 via a similar mechanism. The experiment is described on on page 10 and we have included a discussion of the cIAP1-RIPK2 interaction on page 14/15.



- Figure 5C: what is the extra lower band in the anti-GST blot that is missing in similar blots in Figures 5B and 5E.
The lower band is present also in Figures 5B and 5E although it is weaker in those blots due to lower exposure of the blots. We have performed anti-GST blots on the purified GST-XIAP/cIAP1-BIR2 preparations and in these we observe a band of approx. 26 kDa in addition to a band of the expected size of the recombinant protein (see figure to the right). This is most likely a cleavage fragment of the fusion protein, which we believe is the same signal detected in Figure 5B-5D. We have indicated the band with an asterisk and explain the nature of the detected signal in the accompanying figure legend.

- Figure 5E: Is it pull-down of recombinant proteins in vitro or with the use of cell lysate? Is the "Lysate" labeling correct?
This is indeed a pulldown of recombinant proteins and we have corrected the labelling to “Input” instead of “Lysate”.

- The manuscript sometimes lacks details necessary for the general audience (not experts in the field) to understand the experiments performed. Figures should be labeled better, for example it is not always clear which cells are used without checking figure legends.
We appreciate the reviewer’s comment and agree that the results section in some places lacked sufficient detail about the described experiments. We have included additional information about the experiments shown in figures throughout (pages 4-11). We have also indicated the cell line used in all figure panels where relevant.

- Proof reading for some typos is necessary, i.e. p3 first paragraph " granulatomous pathologies"; p5 second paragraph "ponatinb"
We have proof-read the revised manuscript carefully and any found typos have been corrected.

Additional suggestions that may improve the study:
- Testing new inhibitors in a relevant mouse model of inflammatory disease, where RIPK2 is implicated, would further validate the therapeutic value of the findings.
This is a good suggestion and will be important to further validate the therapeutic value of the CSLP compounds. However, we feel it is beyond the scope of the current study in which we utilise the CLSP compounds primarily to understand the mechanism of action of RIPK2 inhibitory compounds rather than to assess than their therapeutic value.

Referee #2:

Major points:
The manuscript is not within the primary focus of EMBO Journal and is therefore considered to be suitable for a more specialized journal on chemical compounds.
We respectfully disagree with the reviewer. In the study we utilise chemical inhibitors as tools to elucidate fundamental mechanisms for how RIPK2 (and XIAP) facilitate NOD2 signalling. As such, the scope of the study is not solely to characterise chemical compounds but rather to uncover the molecular mechanisms controlling cellular signalling.

Referee #3:

Minor
1) It would be good to describe the classes of kinase inhibitors at the outset. Different classes are referred to in the introduction (and throughout) but for those not in the field it would be helpful to know about the significance of the classes earlier.

We appreciate reviewers suggestion and added a brief description at the outset of our discussion of inhibitors on page 4: “Small molecule kinase inhibitors are categorized into multiple classes, depending on their mode of binding (Roskovski, 2016). This includes type I inhibitors that interact exclusively within the ATP binding pocket, type II inhibitors that bind both to the ATP and an additional back-pocket created when the activation segment of a kinase adopts an inactive conformation, and type III molecules that bind exclusively to this allosteric back pocket. Curiously, we observed that a subset of known RIPK2 inhibitors belonging to different classes displayed potent (nanomolar) cellular activities, including ponatinib (a type II inhibitor) and GSK583 (an ATP-competitive type I inhibitor), and that these molecules also antagonized NOD2-mediated ubiquitination of RIPK2 (Figure 1C; Figure EV1A) (Canning et al., 2015). This implied that the kinase activity of RIPK2 is required for its ubiquitination and, thus, for NOD2 responses.”

2) Please indicate r2 in figure 3d.
We have now indicated R1-R3 in Fig 3D and 3E to make the panels more accessible to the reader.

3) The resolution of the structure is only 3.2A. Because the details of the active site are important it would be helpful to show the electron density for this region and comment on the quality of the map in the main text.
This is a good point raised by the reviewer. We have included the electron densities in Figure EV2E and have included a short comment in the text (page 8): "The structure is at 3.2 A resolution, and the electron density map is of sufficient quality in the region of the inhibitor to place the inhibitor and its relevant functional groups with reasonably good precision (Figure EV2E)."

4) Although the compounds will be described in detail elsewhere it would be helpful to include a simple schematic alongside the crystal structure in Figure 3.
We have included a schematic of CSLP18, CSLP37, CSLP43, CSLP48, and CSLP55 in Figure 3C.

2nd Editorial Decision 11th June 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by two original referees whose comments are shown below.

As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address in a final revision.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

REFEREE REPORTS

Referee #1:

All my concerns were well addressed. I have no further comments.

Referee #3:

All my concerns have been addressed and in my view the manuscript is suitable for publication.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Gyrd-Hansen
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-99372

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	6 animals per group were subjected to treatment as indicated in Figure 4D. Group sizes were initially selected based on power calculations assuming 50% reduction in TNF levels.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were randomly selected into treatment groups from a cohort of age-matched animals. Measurement of readout (ELISA) is objective.
For animal studies, include a statement about randomization even if no randomization was used.	For the animal experiments, animals were randomly assigned to different treatment groups from the cohort of the age-matched wild type animals
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NO
Is the variance similar between the groups that are being statistically compared?	NA

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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Supplier, catalog number and clone number is stated in appendix methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	U2OS/NOD2 cells were generated by M.G-H and are reported in Fiil et al. 2013. THP1 cells were obtained from ATCC and are reported in Hrdinka et al. 2016. HEKBlue and THP1-Blue cells were obtained from Invivogen. RAW264.7 cells. Cell lines have not been authenticated by the authors.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Female 6-8-week-old C57Bl6/J mice purchased from Jackson labs. Animals were housed in Tufts DLAM barrier facility for 2 weeks prior to the experiments under regular day/night cycle and feeding conditions.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were performed in compliance with the ethical standards of animal treatment and according to the protocols approved by Tufts IACUC committee.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Section is inlcuded in manuscript. The structure of RIPK2 in complex with CSLP18 is deposited (PDB ID: 6FU5)
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Source data is included as Appendix Tables
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO